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DOES THIS HAVE GLUTEN? COMPARISON OF GLUTEN-FREE AND UNRESTRICTED DIETS IN INTESTINAL BACTERIAL POPULATIONS AND DIVERSITY

By
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2015

Approved by

Advisor: Professor Colin Jackson

Reader: Professor Marjorie Holland

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ABSTRACT

Bradshaw Daniel Hammond: Does This Have Gluten? Comparison of Gluten-free and Unrestricted Diets In Intestinal Bacterial Populations and Diversity
(Under the direction of Colin Jackson)

As different diets continue to grow in popularity within the United States, the effect of these diets upon bacteria in the intestines is only beginning to be understood. This study focused on the effect that a shift to a gluten-free diet has on the human gut microbiome. Three genetically close subjects were selected for observation over a six month time period. One subject observed a consistent gluten-free diet. A second subject started on an unrestrictive diet, switched to a gluten-free diet for three months, then returned to an unrestricted diet for an additional three months. The third subject left an unrestricted diet. Fecal samples were taken at set time intervals from each subject and bacterial DNA extracted. Bacterial communities were characterized by 16S rRNA gene sequencing. The subject on a consistently gluten-free diet showed a wide variation in bacterial community structure across their samples for the length of the study, while the subject on an unrestricted diet had little variation in their gut microbiome from the start to end of the study. The subject that alternated between diets showed a change in intestinal bacteria when switching to the gluten-free diet, followed by a return to something resembling the initial gut microbiome when they went back to an unrestrictive diet. However, the overriding factor was that each subject showed evidence of a characteristic gut bacterial community. This study shows that a substantial change in personal diet yields a detectable change in intestinal bacterial populations and diversity, which can then be reversed by a removal of the diet. However the individual nature of the gut microbiome means that a change in diet may not necessarily result in a gut bacterial community that resembles other individuals on that same diet.

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Introduction

One environment that holds particular significance to human beings is the human body itself. While not commonly considered by the general public, bacteria inhabit many places on and in the human body. From the epidermis of the skin, to the acidic regions of the stomach, and even the constantly changing landscape of the mouth, bacteria are present throughout our body (Costello et al. 2009). The amount of bacterial species that inhabit the human body is staggering, and they vary from one another as much as different parts of the human body differ from each other. Even within specific parts of the body, changing environmental conditions can select for different species of bacteria to dominate at different times, or under different environmental regimes (Turnbaugh et al. 2009). Some of these changes can be influenced by our own actions, and human beings, however unaware, may have more control over the growth of bacterial species within their body than the growth of plant species in their backyard.

No place within the human body is this idea more prevalent than within the human large intestine. The large intestine is by far the most heavily colonized region of the digestive tract, with up to 10^{12} bacteria per gram of gut contents (Gibson et al. 2010). Largely through the process of fermentation, colonic bacteria are able to both metabolize and produce a wide range of compounds for growth (Gibson et al. 2010). Because the large intestine is so heavily populated and because these bacteria base their metabolism on the food that a human body digests, a diverse bacterial community may be present. Just as the food that can be consumed may vary drastically in a short amount of time, bacteria species can quickly gain or lose their foothold within the intestine. “Blooms” in specific bacterial groups can occur rapidly after a dietary change and these blooms can be reversed by subsequent diet (Walker et al. 2010). The speed at

which bacterial populations might fade and bounce back makes the large intestine an interesting location to observe bacterial community dynamics.

The variable nature of gut bacteria present in any given intestine allows for many different types of comparisons, such as age or geography (Yatsunenko et al. 2012). Individuals can be studied for differences and similarities on a variety of levels. Parameters such as age, gender, geography, or diet can be manipulated for comparison among different individuals or at various lengths of time for the individual themselves. The analysis of the connection between gut bacteria and the human host leads to discoveries toward human wellness (Kinross et al. 2011). Information on problems such as obesity and increased generation of fat has been revealed in part by the study of gut microbiomes (Turnbaugh et al. 2006). Additional studies have led to the discovery of new species within the human intestine and have yielded the largest bacterial genome so far obtained from a human (Lagier et al. 2012).

An important tool for gut microbiome analysis has been the analysis of 16S rRNA sequences. A genotypic method, 16S rRNA analysis is more accurate for identification of bacterial species than observation of phenotypic characteristics (Clarridge 2004). 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains and can lead to the recognition of novel pathogens and noncultured bacteria (Clarride 2004). One of the main benefits of utilizing these 16S rRNA techniques is that they make it possible to study the composition and diversity of intestinal flora without the need for cultivation (Wang et al. 2005). Through the polymerase chain reaction (PCR), fragments of a bacterial sequence can be amplified for study, without the need to produce a culture of the bacterial species for extraction of genetic material. Thus, 16S rRNA gene sequence data is a valuable tool for the identification and comparison of intestinal bacteria for any given number of

individuals under study. This allows for more diverse studies concerning the gut microbiome and has enabled researchers to set forth a variety of parameters for observation.

Within this age of popular diets and the culture of "watching what you eat", the effect of food restrictions upon the bacterial community of the intestinal tract is a field of not only great interest but relevance. One of the dietary choices that is currently popular is the gluten free diet. Originally followed by those who suffer from Celiac disease and gluten intolerance, a gluten free diet has entered the main stream dieting culture and is practiced by many individuals in the United States (Pietzak 2011). This diet seeks to eliminate all sources of gluten from a person's diet, eliminating the consumption of items such as beers, cereals, breads, and most products with origins in wheat (Pagano 2006). The exclusion of many grain-based products from this diet potentially reduces the availability of an important organic molecule for many bacteria; polysaccharides. Polysaccharides are important for the metabolism of many bacterial species, and microbiota of the mammalian intestine likely depend largely on dietary polysaccharides as energy sources (Flint et al. 2008). Moreover, dietary polysaccharides that reach the human large intestine have a major impact on gut microbial ecology and health (Flint et al. 2008). While polysaccharides can be obtained from a variety of plant based food sources including soy, sugar beet, or apples (Van Laere et al. 2000) the majority of polysaccharides in a typical human diet come from grains. As such, an individual who begins a gluten free diet drastically changes the input of these energy rich molecules to their gut bacteria, and this dietary change might be expected to change both the environment and the bacterial community composition of the intestines. Furthermore, individuals who have been on a gluten free diet for a long time (such as those suffering from gluten intolerance or Celiac Disease) could well have gut bacterial

communities that differ substantially from individuals on a more typical diet, as their intestinal environment might select for bacterial populations that use non-grain polysaccharides.

In this study, I monitored the composition of the large intestinal bacterial community of three genetically related individuals on different dietary regimes. One individual had a pre-existing gluten intolerance and had been on a long-term gluten free diet on which they remained for the duration of the study. The second individual had no dietary restrictions and ate what they would normally consume (including gluten containing products). The third individual started on a normal diet that included gluten products but abruptly switched to a gluten free diet, on which they remained for three months before returning to their original diet. Two hypotheses drove the study. The first hypothesis was that a shift from a normal to gluten-free diet would result in a significant change in intestinal bacterial composition, which would be reversed by a return to a normal diet. The second hypothesis was that normal and gluten free diets result in the presence of signature bacterial populations in the large intestine so that when an individual shifts from a normal to gluten free diet, their intestinal bacterial communities would begin to resemble those of an always gluten-free individual. To address these hypotheses I utilized 16S rRNA approaches to determine intestinal bacterial community composition, and bioinformatics approaches to compare this community between the three individuals.

Methods

Sample Collection

Sampling involved three subjects, designated subjects D, B, and J. Subject D took one sample while on their normal diet, then switched to a gluten-free diet for the next three months, continuing to take samples throughout this period (Table 1). After the three month period, Subject D returned to their normal diet and continued to provide samples for an additional month. Subject B normally follows a gluten-free diet. This subject followed the same sampling increments as Subject D, and remained on their usual gluten-free diet for the duration of the study. Subject J followed their normal (not gluten-free) diet over the course of the study, and provided just two samples; one at the beginning and one at the end of the study. Thus, the three subjects included one on a normal diet (J), one on a gluten-free diet over the study period (B), and one who switched to gluten-free and then switched back to a normal diet over the course of the study (D) (Table 1).

Each sample was collected by using a sterile cotton swab to gather fecal matter immediately after defecation. Care was taken to avoid skin contact in order to reduce possible contamination from skin-associated bacteria. Swabs were then placed into a sterile plastic collection tube and frozen (-20 °C) until all samples had been collected.

DNA Extraction and Sequencing

In the laboratory, frozen samples were allowed to thaw to room temperature. Bacterial DNA was then extracted using a Mo Bio PowerFecal DNA Isolation Kit, following the protocol supplied by the manufacturer (Mo Bio Laboratories, Carlsbad, CA). The presence of DNA in

Table 1. Schedule of fecal sample collection for the three subjects (D, B, J) involved in the study. For each sample, the distinction between gluten-free and a normal diet is noted. X represents no sample taken on that sampling date.

Subject	Initial Sample (1)	1 Week (2)	2 Week (3)	1 Month (4)	2 Month (5)	3 Month (6)	1 Week (7)	2 Week (8)	Last Month (9)
D	Normal	Gluten Free	Gluten Free	Gluten Free	Gluten Free	Gluten Free	Normal	Normal	Normal
B	Gluten Free	Gluten Free	Gluten Free	Gluten Free	Gluten Free	Gluten Free	X	X	X
J	Normal	X	X	X	X	X	X	X	Normal

each extraction was confirmed through agarose gel electrophoresis. A portion (V4 region) of the bacterial 16S rRNA gene was then amplified and sequenced using paired-end, barcoded Illumina next generation sequencing (Kozich et al. 2013). The resulting sequence library was then sequenced at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center (UMMC) in Jackson, MS. Sequence data was subsequently downloaded and assessed using the bioinformatics software package, mothur (Schloss et al. 2009) using procedures recommended by Schloss et al. (2011) and Kozich et al. (2013).

16S rRNA Gene Sequence Analysis

The bioinformatics package mothur was used to analyze the data. General processes used and their intended function are summarized in Table 2, but briefly, each sample went through a series of steps to remove potential sequencing errors that prevent accurate analysis. Final sequences were grouped together into operational taxonomic units (OTUs) for diversity analysis, using a 97% similarity criterion (i.e. sequences that were more than 97% similar were regarded as being the same OTU, a surrogate for species). The presence and relative abundance of OTUs within the different samples was then compared, and the similarity scores ordinated through non-metric multidimensional scaling (NMDS) to allow for visual representation of sample similarity.

Table 2. Summary of the various commands within the software package mothur that were used to analyze data in this study, and their intended purpose for analysis.

Command	**Function**
Make.contigs	Initial Processing
Screen.seqs	Screen data for length errors
Unique.seqs	Filters out identical sequences to reduce processing time
Count.seqs	Compress unique sequences and samples together
Alignseqs	Aligns sequences to an established database
Filter.seqs	Filters out non-informative gaps
Pre.cluster	Clusters almost identical sequences together
Chimera.uchime	Identifies chimeras within sequences
Remove.seqs	Removes chimeras
Classify.seqs	Classifies remaining sequences according to GreenGenes
Cluster.split	Groups sequences into operational taxonomic units (OTUs) for diversity analyses
Make.shared	Determines how many times sequences classified as a particular operational taxonomic unit (OTUs) were found in each sample
Count.groups	Checks how many sequences are within each sample
Classify.otu	Identifies operational taxonomic units (OTUs)
Dist.shared	Creates a similarity matrix, based on presence and/or abundance of operational taxonomic units (OTUs)
NMDS	Allows for ordination of samples for comparisons

Results

DNA was successfully extracted from all samples, as verified by agarose gel electrophoresis (Figure 1). Following DNA sequencing, a total of 1,224,284 valid bacterial 16S rRNA gene sequences were collected across all subjects (Table 3). The majority of bacterial sequences obtained classified within the phylum Firmicutes which accounted for 995,267 (81%) of the total number of sequences obtained. Within this phylum, sequences identified belonging to the class Clostridia were the most prevalent, accounting for 963,820 (79%) of the total dataset (Table 4). *Blautia* and *Faecalibacterium* were the abundant genera within this class, accounting for almost 16% and 11%, respectively of all sequences obtained (Table 2). *B. obeum* was the most prevalent species of *Blautia* while *B. producta* was the second most prevalent. A third sequence type was identified within this genus, however it could not be classified to a particular species and so was labeled as *Blautia* unclassified. *F. prausnitzii* was the only detected species of *Faecalibacterium*, accounting for all sequence types of that genus (Table 4).

The second most common phylum after the Firmicutes was the Bacteroidetes, which accounted for 8% of the sequences obtained, with genus *Bacteroides* being the most prevalent. *B. ovatus* was the most detected sequence within that genus, although it only accounted for 0.7% of the total sequences obtained (Table 5). The next most common phylum was Actinobacteria (4.8% of all sequences), of which approximately half of the sequences belonged to the genus *Bifidobacterium* (2.4% of the total), with the species *B. adolescentis* constituting the largest portion (Table 5). Represented at around the same relative frequency of Actinobacteria was phylum Verrucomicrobia

Figure 1. Confirmation of the presence of DNA within each sample by agarose gel electrophoresis. DNA was extracted from Subject B for all samples (upper row, slots 1-6), Subject D for all samples (upper row, slots 7-15), and Subject J for both samples (upper row, last slot, lower row, first slot).

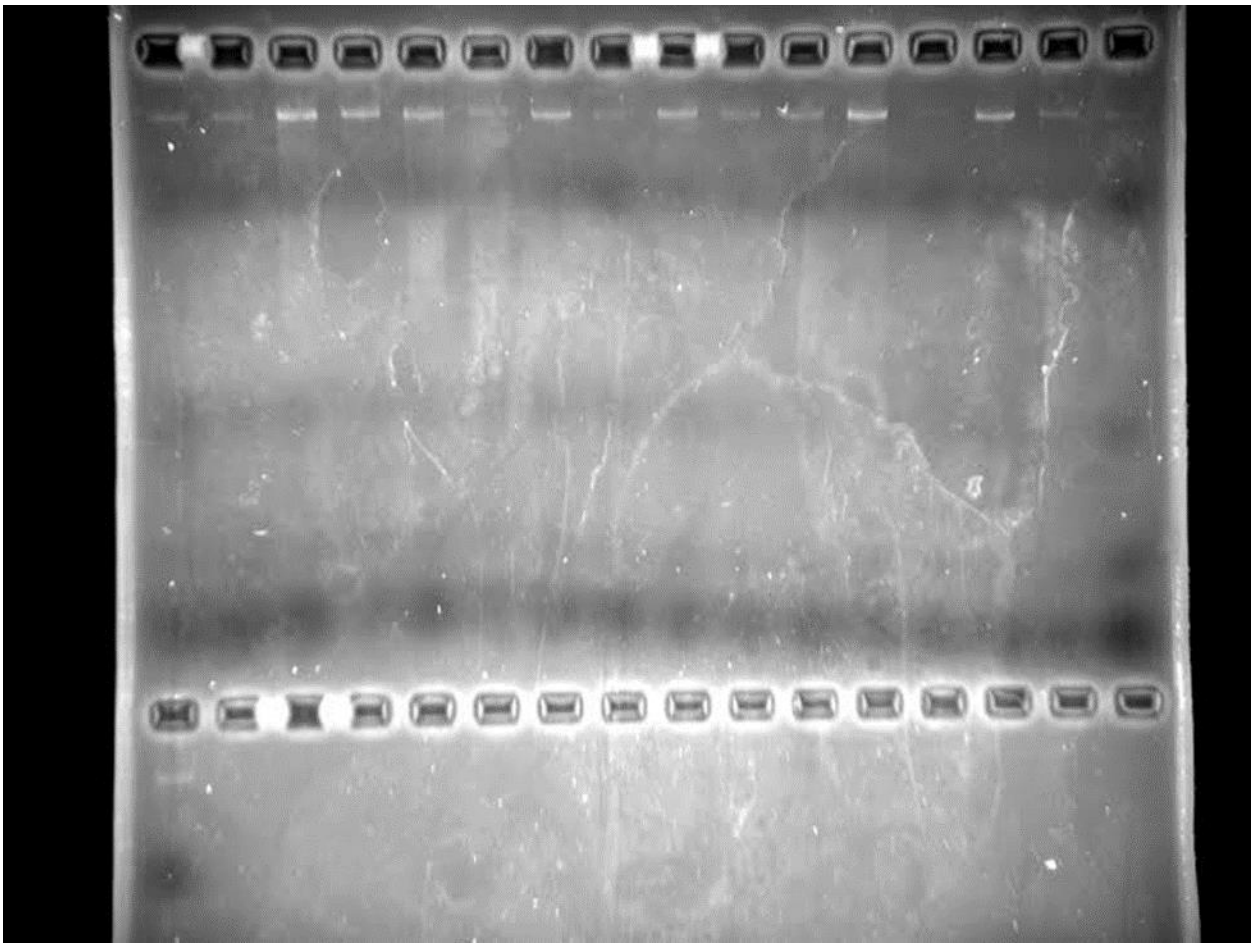


Table 3. General overview of the bacterial phyla present and their relative percentage of the total bacteria sequences gathered from fecal samples obtained from the three subjects. The five most prevalent phyla (Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia) have their total sequences and relative percentages highlighted red to signify their abundance in regard to the other phyla.

Taxon	Total	Percent
k__Bacteria	1224284	100
p__Bacteroidetes	98825	8.1
p__Chlorobi	18	0.0014
p__Chloroflexi	2	0.0001
p__Cyanobacteria	942	0.08
p__Firmicutes	995267	81.3
p__Fusobacteria	16	0.0013
p__Acidobacteria	16	0.0013
p__Actinobacteria	58522	4.8
p__Lentisphaerae	1	0.0001
p__Nitrospirae	13	0.0011
p__Planctomycetes	78	0.0064
p__Aquificae	3	0.0002
p__Proteobacteria	11681	0.95
p__Synergistetes	2	0.0002
p__TM7	22	0.002
p__Armatimonadete	6	0.0004
p__Tenericutes	7	0.0005
p__Verrucomicrobia	58413	4.8

Table 4. Representation of the Firmicutes and its genera and species in the total sequences collected from fecal samples of all three subjects. The class Clostridia, to which all the genera in the table belong, has been highlighted red for its relative abundance. Within the class Clostridia, the genera *Blautia* and *Faecalibacterium* have also been highlighted red to signify their importance. The two most prevalent species (*Blautia obeum* and *Faecalibacterium prausnitzii*) within the two most bountiful genera have also been highlighted red.

Taxon	Total	Percent
k__Bacteria	1224284	100
p__Firmicutes	995267	81.3
c__Clostridia	963820	78.7
g__Roseburia	52773	4.3
g__Ruminococcus	2855	0.2
g__Shuttleworthia	1	0.0001
g__[Ruminococcus]	6609	0.54
unclassified	93819	7.7
g__Blautia	192834	15.8
s__obeum	31577	2.6
s__producta	1039	0.1
unclassified	160218	13.1
g__Clostridium	32913	2.7
g__Coprococcus	68623	5.6
g__Oscillospira	8900	0.7
g__Ruminococcus	48427	4.0
g__Subdoligranulum	3014	0.2
unclassified	41587	3.4
g__Anaerotruncus	29	0.002
g__Butyricicoccus	1642	0.1
g__Clostridium	42	0.003
g__Faecalibacterium	134067	10.95
s__prausnitzii	134067	10.95

Table 5. Representation of the phyla Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia along with their most prevalent species in sequence data collected from the fecal samples of all three subjects. Each phylum is highlighted red for clarification. The subdivisions directly underneath a phylum are present within the above phyla.

Taxon	Total	Percent
k__Bacteria	1224284	100
p__Bacteroidetes	98825	8.1
s__ovatus	9014	0.7
p__Actinobacteria	58522	4.8
g__Bifidobacterium	28956	2.4
s__adolescentis	27972	2.3
p__Proteobacteria	11681	1.0
c__Gammaproteobacteria	7263	0.6
p__Verrucomicrobia	58413	4.8
g__Akkermansia	58387	4.8
s__muciniphila	58387	4.8

(4.8% of the total). Genus *Akkermansia* accounted for almost all of the sequences within this phylum (4.8% of the total) especially the species *A. muciniphila* (Table 5). The least represented of the more numerous phyla was the Proteobacteria (1% of the total), with Gammaproteobacteria the most prevalent subphylum (Table 3). Genus *Escherichia* was the most prevalent genus within the *Gammaproteobacteria*, with *E. coli* making up the largest proportion of identified *Escherichia*.

Subject B possessed the highest proportion of phylum Bacteroidetes of any subject, particularly within the third, fourth, and fifth samples (Figure 2). However, during the sixth sample for subject B, the proportion of Bacteroidetes in the total sharply dropped, approaching levels more similar to the other subjects' samples. Other than samples B3, B4, and B5 the levels of genus *Blautia* and species *B. obeum* were relatively similar between all the samples of the three subjects (Figure 3). The other common species, *F. prausnitzii*, showed more variation between subjects (Figure 4). On average, subject B presented lower levels of *F. prausnitzii* than the other subjects. Subjects D and J showed closer levels, though Subject D did have a spike of the species during the eighth and ninth samples (Figure 4). The species *A. muciniphila* varied sharply between samples for all three subjects (Figure 5). Subjects B and D showed a pattern of the prevalence of this species sharply rising within one sample and proceeding to drop off in the next sample or two before sharply dropping off. Subject J showed a similar decline, albeit at a lower level. The Gammaproteobacteria were present at low levels for nearly all the samples among the three subjects (Figure 6). However, the eighth sample of Subject D showed a sharp increase which then dropped in the next sample.

Figure 2. Presence of Phylum Bacteroidetes (blue bars) and species *Bacteroides ovatus* (orange bars) collected from fecal samples of three subjects. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was initially on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

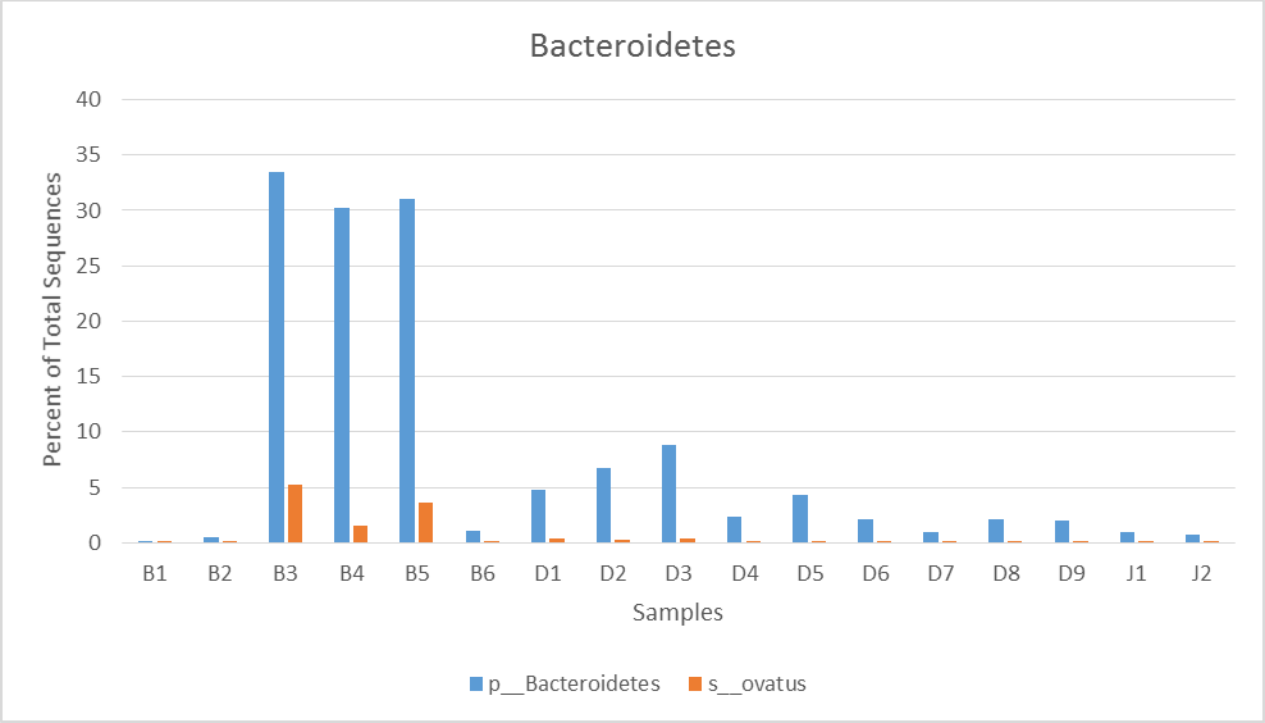


Figure 3. Presence of genus *Blautia* (blue bars; within phylum Firmicutes) and species *B. obeum* (orange bars) collected from fecal samples of three subjects. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

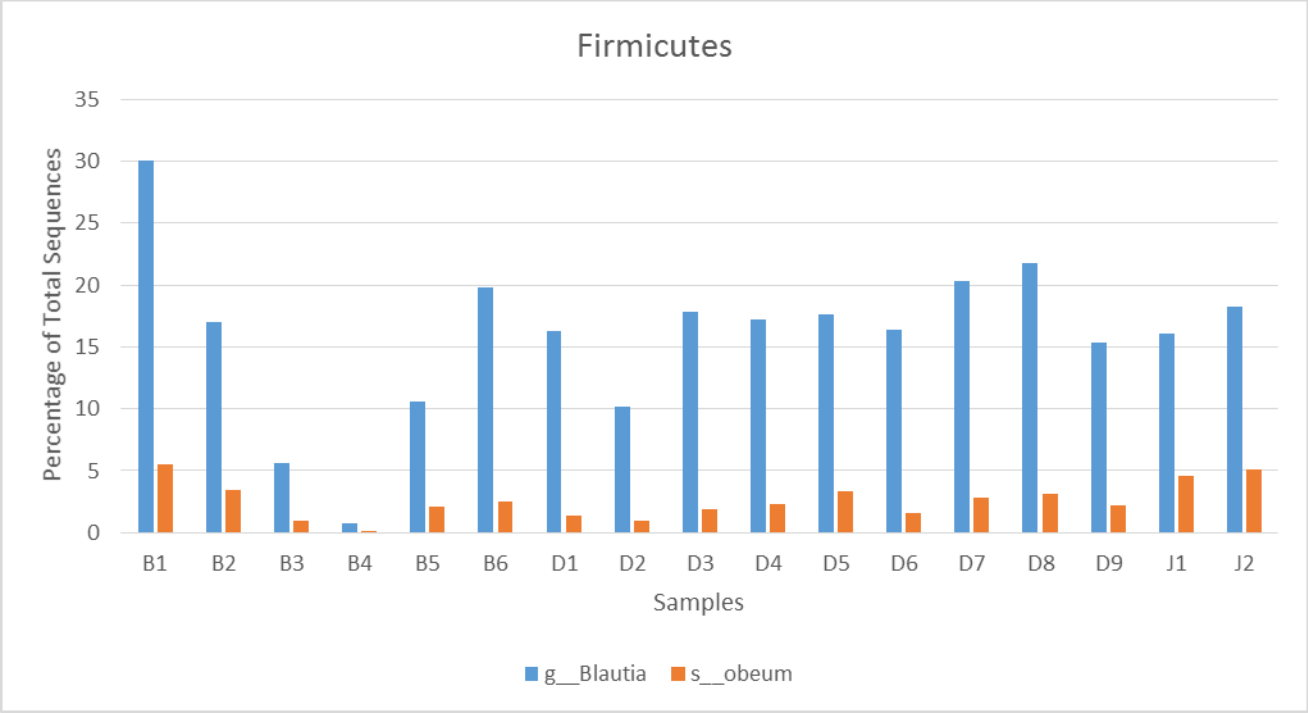


Figure 4. Presence of species *Faecalibacterium prausnitzii* collected from fecal samples of three subjects. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

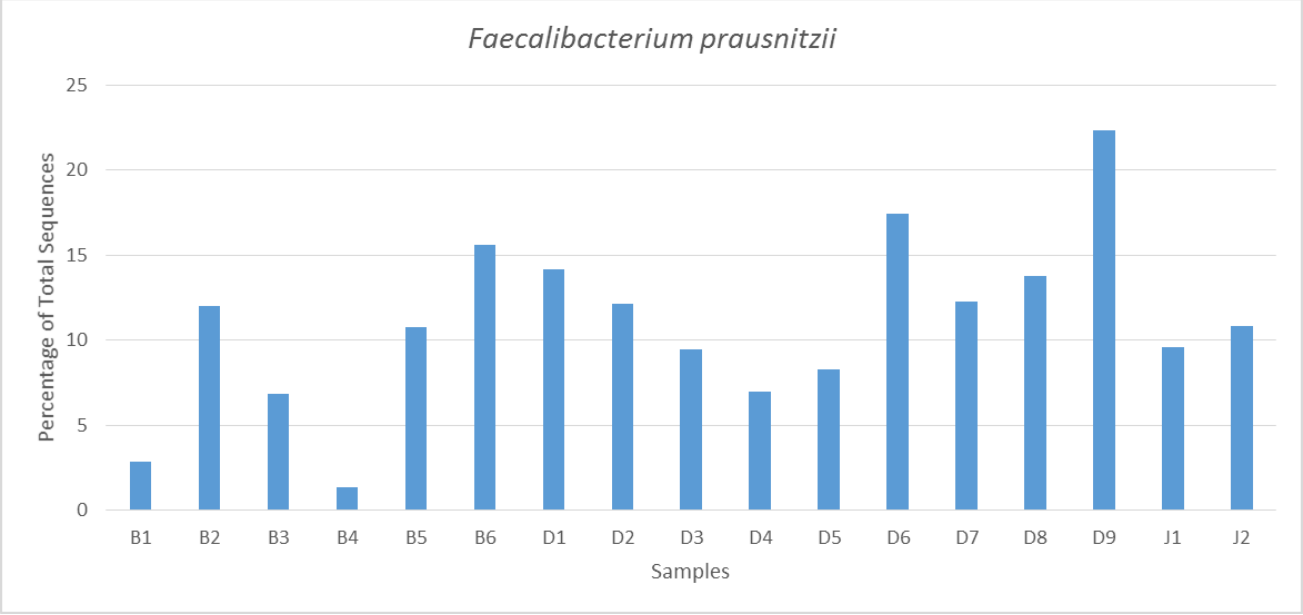


Figure 5. Presence of species *Akkermansia muciniphila* collected from fecal samples of three subjects. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

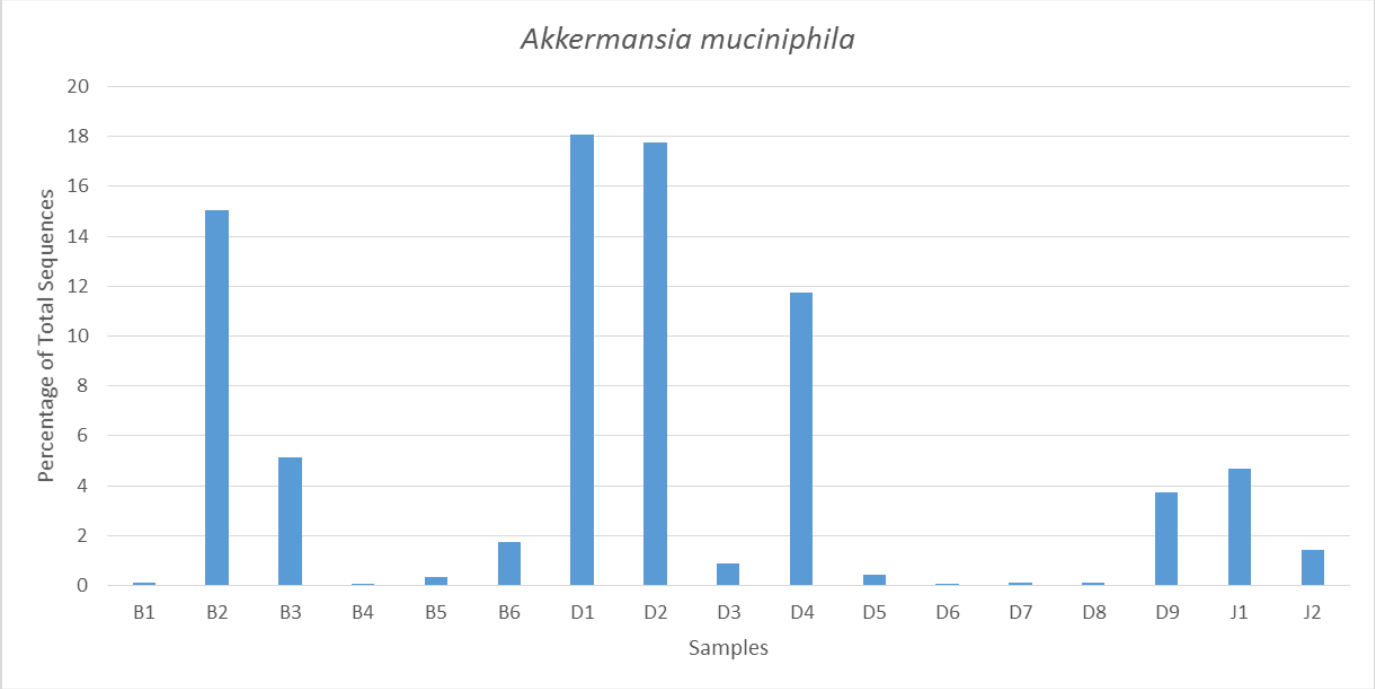


Figure 6. Presence of phylum Proteobacteria (blue bars) and class Gammaproteobacteria (orange bars) collected from fecal samples of three subjects. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

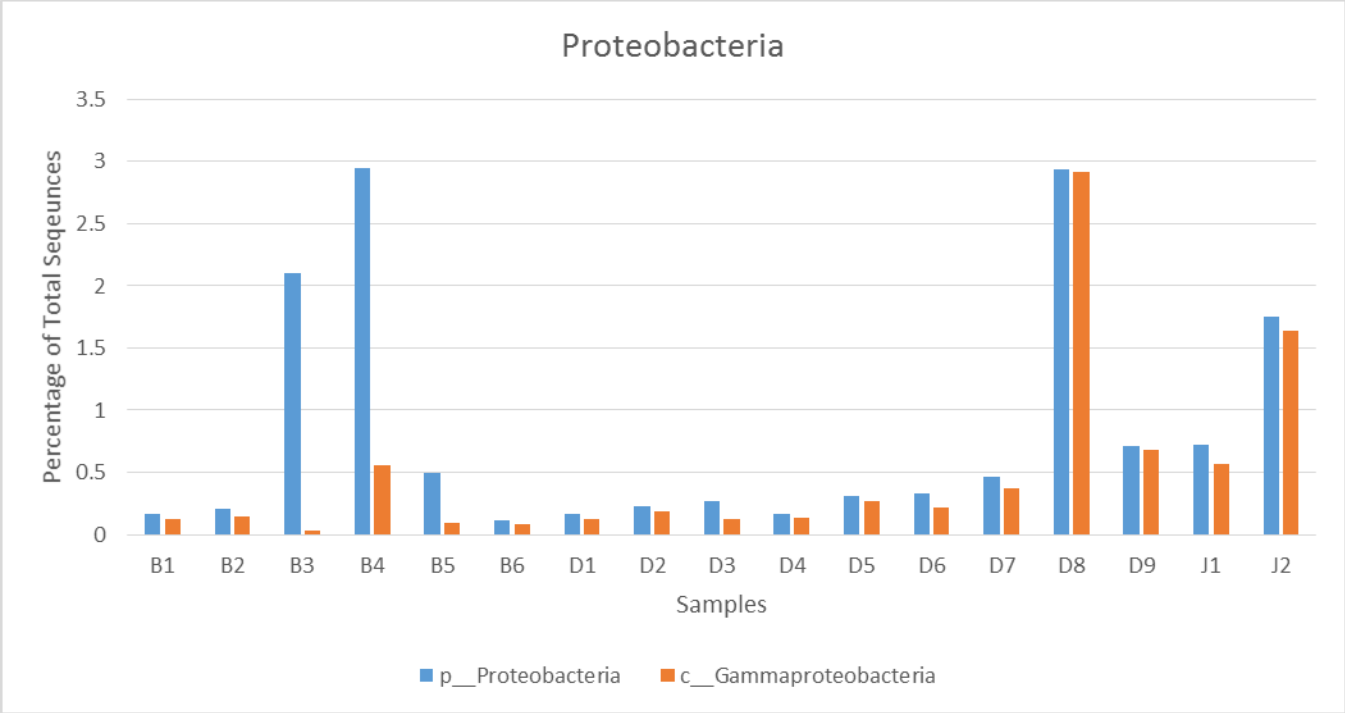
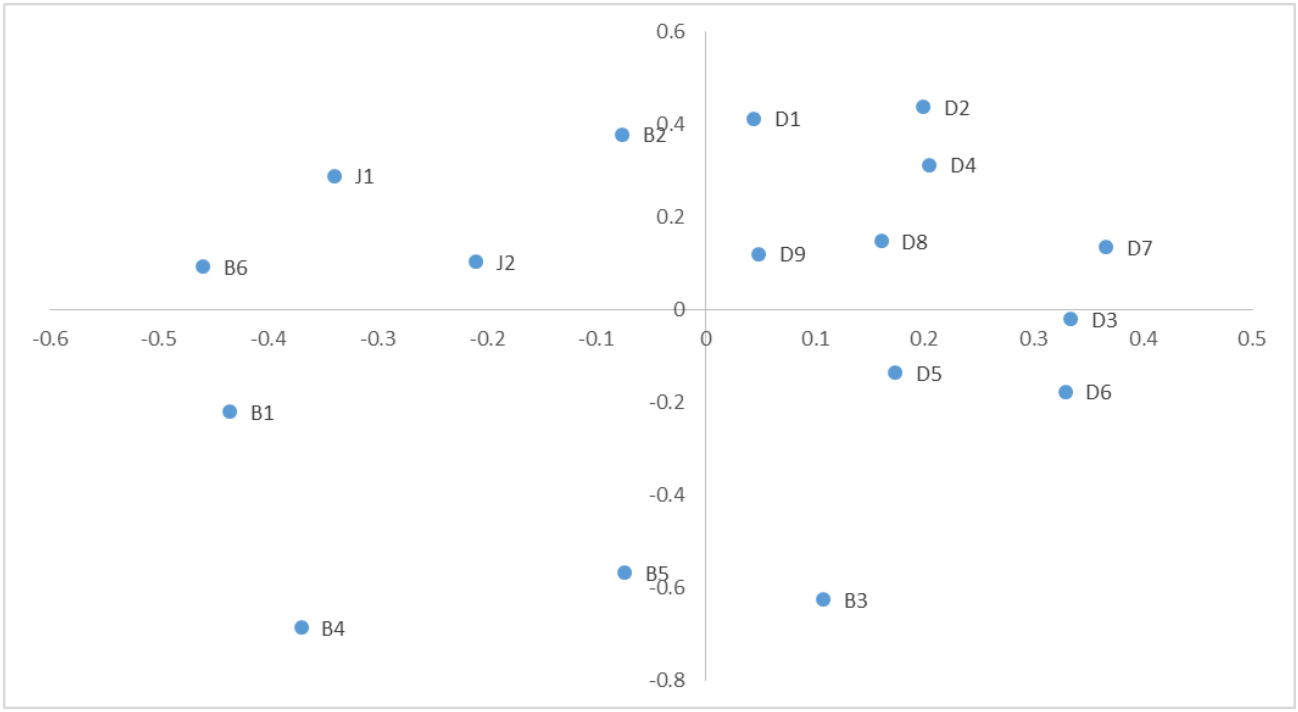


Figure 7. NMDS ordination based on theta similarity scores of bacterial communities in fecal samples taken from three subjects (B, D, J) over a 6 month period. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.



Similarity between samples was analyzed using the theta similarity index, which was subsequently ordinated using NMDS (Figure 7). The two samples from Subject J had the closest clustering of any of the three subjects. Different samples from Subject D were more spread out but still tended to cluster along the positive side of the first axis. In contrast, communities from Subject B were located towards the negative end of axis 1 and also showed the greatest amount of variation of the three subjects. Subjects B and J were in closer proximity to each other than Subject D was to either. In terms of influence of dietary change, subject D's samples generally drifted away from the first sample, and after reverting to their original diet (after sample D6), began to drift back toward the initial sample taken.

Similar patterns were evident when communities were compared using the Jaccard index, which looks solely at the presence or absence of specific bacterial taxa rather than their relative proportions (Figure 8). However, in this case the position of subjects B and D on the first axis was reversed, and the various samples from Subject B (especially B2-6) tended to be more clustered than in the NMDS ordinations derived from the theta index. Subject D again showed variability between each sample. The first and second samples (D1 and D2) were the furthest apart between of any consecutive samples. Samples D3-6 drifted closer to the first sample before spreading out in the seventh and eighth samples. The last sample (D9) marked a return toward the first sample (Figure 8).

The most prevalent operational taxonomic units (OTUs) in the dataset came from the phylum Firmicutes, with the genera of *Blautia*, *Faecalibacterium*, *Roseburia*, *Peptoniphilus*, and *Ruminococcus* being well represented (Table 6). One OTU that was a member of Phylum Bacteroidetes was relatively abundant and classified as the genus

Figure 8. NMDS ordination based on Jaccard similarity scores of bacterial communities in fecal samples taken from three subjects (B, D, J) over a 6 month period. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period.

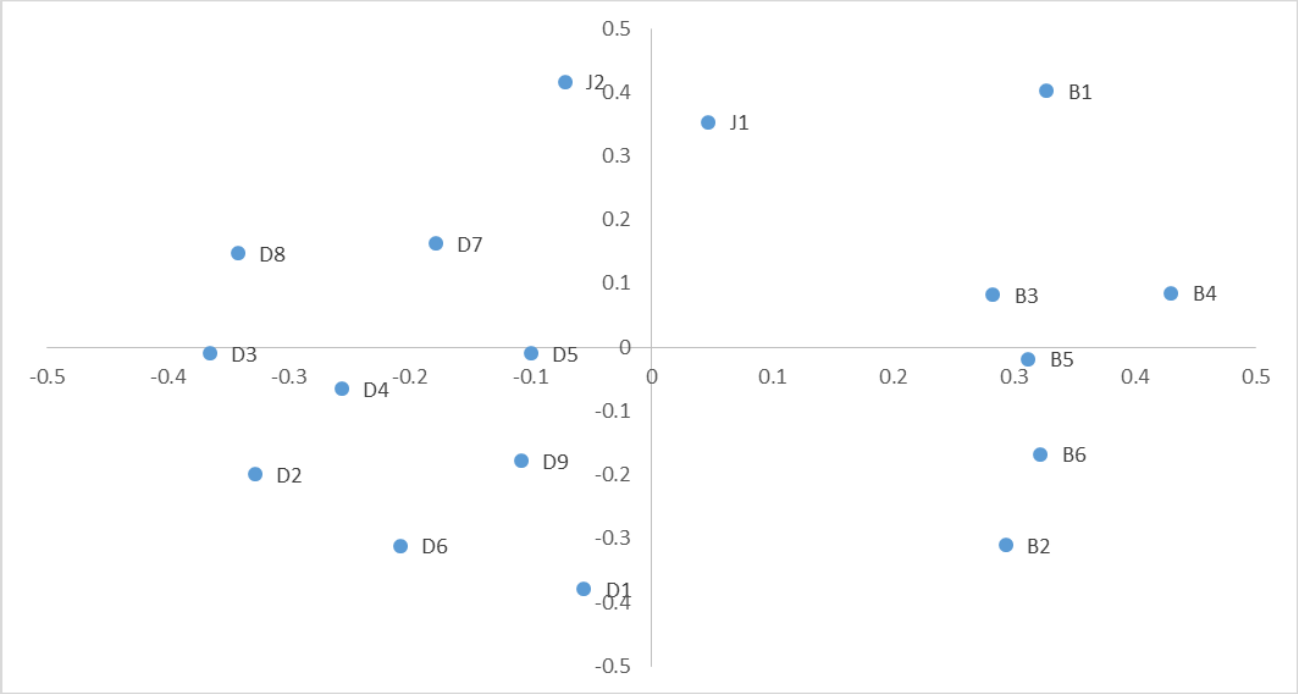


Table 6. Most abundant operational taxonomic units (OTUs) in samples taken from three subjects (B, D, J) over a 6 month period. Numbers after letters indicate the sampling order. Subject B was on a gluten-free diet, subject D was on a regular diet (D1) but switched to gluten-free (D2-D6) and then returned to a regular diet (D7-D9), subject J was on the same consistent regular diet over the study period. If two samples share the same OTU, they are grouped together in the same line separated by a comma. The last column identifies the percentage of the total bacterial reads in that sample that were accounted for by each OTU, if two samples shared the same OTU they are separated by a comma.

Sample	OTU	Taxonomy	% of Total
B1	Otu0002	p__Firmicutes(100);g__Blautia(100);unclassified(100);	24.5
B5, D6	Otu0003	p__Firmicutes(100);Faecalibacterium(100);s__prausnitzii(100);	10.8, 17.5
D5	Otu0004	p__Firmicutes(100);g__Blautia(100);unclassified(100);	14.2
D3	Otu0005	p__Firmicutes(100);g__Roseburia(100);s__faecis(94);	8.5
D9	Otu0006	p__Firmicutes(100);g__Faecalibacterium(100);s__prausnitzii(100);	22.3
D7, D8	Otu0008	p__Firmicutes(100);g__SMB53(100);unclassified(100);	16.6, 14.8
B4	Otu0010	p__Firmicutes(100);g__Peptoniphilus(100);unclassified(100);	19.3
J1, J2	Otu0014	p__Firmicutes(100);f__Ruminococcaceae(100);unclassified(95);unclassified(95);	12.0, 9.6
B3	Otu0031	p__Bacteroidetes(100);g__Bacteroides(100);unclassified(100);	8.4
B2, D1, D2, D4	Otu0032	p__Verrucomicrobia(100);g__Akkermansia(100);s__muciniphila(100);	15.0, 18.1, 17.8, 11.7
B6	Otu0056	p__Firmicutes(100);g__Ruminococcus(100);unclassified(100);	16.8

Bacteroides. An OTU identified as *A. muciniphila* from the phylum Verrucomicrobia was also relatively abundant in some samples (B2, D1, D2, and D4). Subject J had the same dominant OTU (a member of Phylum Firmicutes, Family Ruminococcaceae) for both samples.

Discussion

The phyla Firmicutes and Bacteroidetes were the most common bacterial phyla present from all three subjects. This is unsurprising as gut microbiota in adults are dominated by these two phyla (Ley et al. 2006). Firmicutes are Gram-positive, sometimes anaerobic bacteria, and the most prevalent sequences were identified as being in the genus *Faecalibacterium*. The large representation of this typical intestinal genus supports the fact that bacterial samples were taken from the intestine and not the outside skin of the anus (Miquel et al. 2013). Sequences identified as being in the phylum Proteobacteria were also prevalent, with the class of Gammaproteobacteria being the most common. Most of the sequences in the Gammaproteobacteria were within the family Enterobacteraceae, which is known to contain many species of bacteria found within human intestines (Rajilic-Stojanovic et al. 2007). Specifically, one bacterium from this family that was found within all three subjects was *Escherichia coli*. *E. coli* are well adapted to the conditions of the human large intestine (Rajilic-Stojanovic et al. 2007) and again, the presence of these taxa supports the case of a correct sampling and handling procedure.

Comparisons of overall community similarity revealed a pronounced variation between subjects. Surprisingly, subjects B and D, which were both observing a gluten-free diet for most of the study, showed the largest gap in gut bacterial community structure between any two subjects. This can possibly be explained by the fact that while all three subjects were genetically similar, meal decisions were left up to the subject's personal preference. This allowed for a large variation in diet not only between each subject, but also for a single subject at various points during the study.

Subject B, a college student, lived in a town with a wide spectrum of cuisine possibilities and frequently ate out at multiple locations offering different styles of food. Previous studies have shown that diet can partially modulate gut microbiome composition (David et al. 2014), and a controlled-feeding study of ten subjects showed that microbiome composition changed detectably within 24 hours of initiating drastically different food intake (Wu et al. 2011). This could explain the high variability of Subject B's gut bacterial community, as evidence by variation between sample points in NMDS plots. Subject B also showed more taxonomic variability in the most abundant bacterial types (OTUs) present in each sample, with dominant bacterial populations from three distinct phyla (Firmicutes, Verrucomicrobia, and Bacteroidetes), compared with two for Subject D (Firmicutes, Verrucomicrobia) and just one phylum for Subject J (Firmicutes). This taxonomic variation again supports the assumption that a constantly varied diet produces a broader range of microbiota (David et al. 2014). Even within the phylum Firmicutes, Subject B showed the most variation, and no two samples had the same OTU as the most abundant.

In contrast Subject D, a head of a household, tended to eat more at home, likely having a more stable and consistent diet. This dietary consistency could be reflected in lower variation for that subject's gut community and Subject D provided the least variation in OTUs, with multiple samples yielding the same dominant OTU. Subject D also showed less overall variation between sample dates (when on their regular diet) than Subject B. Subject D altered their diet from their regular one to a gluten-free one and this change did lead to a shift in the structure of the gut bacterial community, at least as suggested by the NMDS ordinations. Samples D2 and D3 reflect a shift to a gluten-free diet and were among the most different from D1 (regular diet), based upon their distance in NMDS plots from that sample. This trend continued in subsequent samples (D4,

D5 and D6), with D6 marking the last sample taken while on the gluten-free diet. After returning to their regular diet, subsequent samples (D7, D8, and D9) suggest a community that is drifting back to the initial one, and the final sample taken is quite similar to the original one, likely marking the return to normalcy for this subject. A change in intestinal bacteria following a pronounced dietary change (and the subsequent reversion after changing back) has been seen within other studies, and blooms in specific bacterial groups occurred rapidly after a change in dietary fiber compositions and these were rapidly reversed by the subsequent diet (Walker et al. 2010).

Prior differences in intestinal bacterial communities have been observed between adults on gluten-free and normal diets (Nistal et al. 2012). That study sampled duodenal sections from three groups of adults: healthy, those untreated for Celiac Disease, and those treated for Celiac Disease. Similar to Subject D for a portion of this study, the healthy group was allowed an unrestrictive diet, while the group treated for Celiac Disease was put on a gluten-free diet- similar to Subject D during other portions of this study. The overarching bacterial populations for each group were then analyzed and mapped. The subsequent map revealed that both groups possessed widely different populations from each other (Nistal et al. 2012); patterns that are similar to the different phases of Subject D within this study, as the gut bacterial communities for Subject D during the gluten-free and unrestricted phase were certainly different. Another similarity of that prior study to the current one is that, at a finer taxonomic resolution, the dominant OTUs of the healthy and celiac groups were different (Nistal et al. 2012).

The presence or absence of gluten has previously been found to affect intestinal bacteria, as shown from a study that had volunteers collect fecal samples before and after a one month period of gluten-free diet (De Palma et al. 2009). None of those subjects were celiac or gluten-

free before the study. That study found a noticeable reduction in polysaccharide intake during the gluten-free period, a predicted outcome of a switch to a gluten-free diet (De Palma et al. 2009). The change in bacterial communities between diets for the volunteers reflect a similar change presented by Subject D, and the study concluded that a gluten-free diet may influence the composition and immune function of the gut microbiota in healthy individuals (De Palma et al. 2009).

Subject J, a high school student, represented an intermediate between the other two subjects in terms of dietary choices, having the stability of a household food source, while also possessing the mobility of occasionally eating out. This can be seen within the NMDS ordinations, as Subject J's gut microbiota fell between Subject B and D, being similar, yet distinct, from both. However, Subject J had just two samples taken over the course of the entire study (the subject was sampled as just an additional reference point) so patterns are difficult to determine. While both samples of Subject J yielded the same dominant OTU (within Phylum Firmicutes), suggesting low variation between samples, it is possible that more variation in the gut community would be apparent if additional samples were taken. However, these two samples were taken almost six months apart, which does suggest a stable gut community, regardless of the low number of samples.

At the start of this study, two hypotheses were made regarding potential changes in gut bacterial community composition. The first hypothesis predicted that a shift from a normal to gluten-free diet would result in a significant change in intestinal bacterial composition, which would be reversed by a return to a normal diet. To some extent, this was confirmed as Subject D showed this pattern in their gut community. The divergence of the gluten-free samples from the initial (regular diet) sample and the convergence of the normal diet samples to the initial sample

verified this hypothesis. However, the second hypothesis that normal and gluten free diets would result in the presence of signature bacterial populations in the large intestine was not proven, as when Subject D shifted from a normal to a gluten free diet, their intestinal bacterial communities did not appear to resemble those of the always gluten-free Subject B. Rather, each subject (including Subject J) appeared to have their own, somewhat consistent, gut microbial community. Others have observed that while a subject may alter their bacterial communities through dietary change, a subject's overarching "enterotype" identity remains largely the same (Wu et al. 2011). The intestinal environment of the human intestine is under a constant plethora of factors that affect the growth of the bacterial populations that live there. While shifting ones diet from normal to gluten-free may seem like a radical change, this study suggests that this one variable, while an important one, may not be significant enough by itself to predictably alter the complete environment of the human intestine.

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